

Immediate Pigment Darkening Phenomenon. A Reevaluation of Its Mechanisms*

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Proposed mechanisms of immediate pigment darkening (IPD) are controversial. They include photooxidation of "premelanin," changes in the distribution pattern of microfilaments and microtubules, movement of melanosomes to melanocyte dendrites, increased transfer of melanosomes to keratinocytes, and changes in the melanosome distribution pattern in keratinocytes. We investigated the following aspects of IPD: (1) production of IPD by UVA under physiologic and nonphysiologic conditions in full-thickness skin and epidermal sheets; (2) reversibility of IPD in vitro after in vivo and in vitro production; (3) blocking of IPD by disruption of the microfibrillar or microtubular system in vitro; (4) alterations of the cytoskeleton of melanocytes; (5) the melanosome distribution pattern in melanocytes and keratinocytes.

The results were as follows: IPD could be elicited in vitro in full-thickness skin and in epidermal sheets. Its pro-

duction was temperature independent (0° – 37°C) and was not inhibited by repeated freezing and thawing, or by formalin fixation. IPD was reversible in vitro under tissue culture conditions but only in viable skin. IPD could not be blocked by substances that disrupt the microfibrillar or microtubular system (cytochalasin B, colcemid, vincristine). As shown with a monoclonal antivimentin antibody, IPD-producing UVA doses did not induce changes in the cytoskeleton of melanocytes. No changes in number and distribution pattern of melanosomes were observed electron-microscopically and by morphometric analysis of EM micrographs. Production of IPD does not depend on the structural and functional integrity of the melanocyte cytoskeletal apparatus and is not confined to viable skin, whereas its reversibility is. The fact that no increased melanosome transfer occurs may explain the lack of a UV protective action. *J Invest Dermatol* 87:648–652, 1986

Immediate pigment darkening (IPD), also called immediate tanning, is a transient grayish-brown discoloration of the skin that develops immediately after exposure to UV radiation or sunlight and fades gradually within a few hours [1,2]. It is best seen in subjects with darker skin color and on previously sun-exposed skin areas. The action spectrum of IPD lies in the UVA (320–400 nm) and in the visible light region (>400 nm) [3–5]. With the use of high-intensity UVA radiation sources for the therapy of skin diseases, IPD has again attracted attention. Its physiologic function, if any, is unknown, and no photoprotective action has been convincingly documented.

The hypothetical mechanisms of IPD are still controversial and include: (1) redistribution of melanosomes and of intermediate (100 Å) filaments in the melanocytes from a perinuclear location to the distal portions of the dendrites; (2) increased transfer of melanosomes into keratinocytes; (3) redistribution of melanosomes within keratinocytes [6–8]; and (4) photooxidation of "premelanin" [9]. In the present study we investigated several aspects of IPD with the aim of reevaluating these proposed mechanisms.

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Abbreviations:

- IPD: immediate pigment darkening
- IR: infrared
- MEM: minimal essential medium
- PBS: phosphate-buffered saline

We first tried to determine the conditions under which IPD can be produced in human skin in vitro and whether its production can be blocked by various procedures. To this end we have tested IPD production by irradiation with UVA, UVB, and infrared (IR) of skin specimens under physiologic tissue culture conditions and under various nonphysiologic conditions. We then examined the possible morphologic changes that were previously believed to represent the ultrastructural substrate of IPD production and to be responsible for the visible skin coloration of human skin in vivo.

MATERIALS AND METHODS

In Vitro Studies

Skin Specimens: Shave biopsies were obtained from usually sun-exposed skin (mostly lower back) of normal human volunteers whose ability to develop IPD had been confirmed by previous test exposures to UVA radiation. The specimens were placed in tissue culture dishes and allowed to float on phosphate-buffered saline (PBS) with the horny layer remaining outside the fluid. For some experiments epidermal sheets were prepared by sodium bromide incubation (37°C , 2 h) and were also put on PBS. Both full-thickness skin and epidermal sheets were then subjected to the experimental procedures detailed below.

UVA Exposure: For UVA irradiation we used a Waldmann PUVA 200 unit (H. Waldmann, GMBH, Werk für Lichttechnik, Schwennigen, F.R.G.) equipped with Sylvania F8T5 PUVA fluorescence bulbs, which emit broad-band UVA from 320–400 nm with a maximum at 360 nm (irradiance at the specimen level as measured with a Waldmann PUVA-Meter: 7.7 mW/cm^2). The temperature within the unit was kept at 30° – 32°C throughout the

entire irradiation period. Some specimens were irradiated while floating on ice-cooled PBS. The irradiations used ranged from 25–40 J/cm².

UVB Exposure: For UVB irradiations an Osram Ultra-Vitalux-lamp (Osram, GMBH, Munich, F.R.G.) [irradiance at the specimen level as measured with an IL 700 Research Radiometer (Newburyport, Massachusetts) at 297 nm: 0.54 mW/cm²] served as radiation source. The irradiations used in these experiments ranged from 50–100 mJ/cm².

Infrared Exposure: For IR irradiation a Sollux 760 (Heraeus, Original Hanau, F.R.G.) IR source was used. Since we were unable to measure IR radiation more accurately, we measured the temperature in the tissue culture dish. The temperatures produced ranged from 30°C up to 60°C. Exposure times ranged from 15–30 min.

Controls: Nonirradiated skin specimens obtained from adjacent skin areas were used as controls. For each experiment the color of the irradiated specimen was compared with that of a nonirradiated one of the same subject.

Immediate Pigment Darkening Blocking Experiments: In order to investigate the effect of disintegration of the cytoskeleton on IPD production the following agents were added to culture fluid (PBS) 2 h prior to and throughout UVA exposure: (1) 10 µg/ml cytochalasin B (Aldrich Chemical Corp., Milwaukee, Wisconsin), which disrupts microfilaments [10,11]; and (2) 5 µg/ml colcemid (Gibco, Grand Island, New York) and (3) 10 µg/ml vincristine (Eli Lilly Corp., Giessen, F.R.G.), which cause microtubule disassembly [12]. In order to test IPD production in damaged skin the biopsy specimens were either fixed for 15 min, 30 min, 5 h, and 16 h with 5% formalin, or underwent freezing and thawing 5 times prior to UVA exposure.

Reversibility Tests: The reversibility of IPD in vitro was investigated by incubation of IPD-positive skin and of IPD-positive formalin-fixed and frozen and thawed skin for 48 h and 31°C in minimal essential medium (MEM) in the dark.

In Vivo Studies

Skin Specimens: Immediate pigment darkening was produced in normal human volunteers with UVA irradiations ranging from 40–100 J/cm² delivered by an irradiation system as above. Biopsy specimens were secured immediately after exposure and processed for indirect immunofluorescence and electron microscopy. Nonirradiated adjacent skin served as control.

Immunofluorescent Investigations: For the detection of gross alterations of the intermediate filament (vimentin) compartment of the cytoskeleton of melanocytes, an indirect immunofluorescence method was used. Epidermal sheets of IPD skin and control skin were prepared with 0.5 M ammonium thiocyanate (20 min; 37°C), fixed for 20 min in acetone (20°C), rinsed with PBS (1 h; 20°C), and incubated for 2 h at 37°C with a mouse monoclonal antivimentin antibody (VIC-C2) [13] 1:50 in PBS. After rinsing with PBS the sheets were exposed to a fluorescein isothiocyanate (FITC)-labeled goat-antimouse IgG antibody (Tago Inc., Burlingame, California) for 1 h at 37°C. Finally, to identify Langerhans cells which also contain antivimentin, double staining with a rhodamine-labeled anti-HLA-DR antibody (Becton Dickinson, Monoclonal Center Inc., Mountain View, California) was performed. The sheets were then spread on glass slides and examined with a Leitz Ortholux II fluorescence microscope.

Electron Microscopy and Morphometric Analysis: Biopsy specimens of IPD skin and control skin were fixed in Karnovsky's fixative and processed according to routine procedures. For morphologic investigation, ultrathin sections were examined with a Philips EM 400 electron microscope at 80 kV. For the morphometric analysis, ultrathin sections were cut perpendicular to the skin surface and photographed part by part at a primary magnification of 8000×,

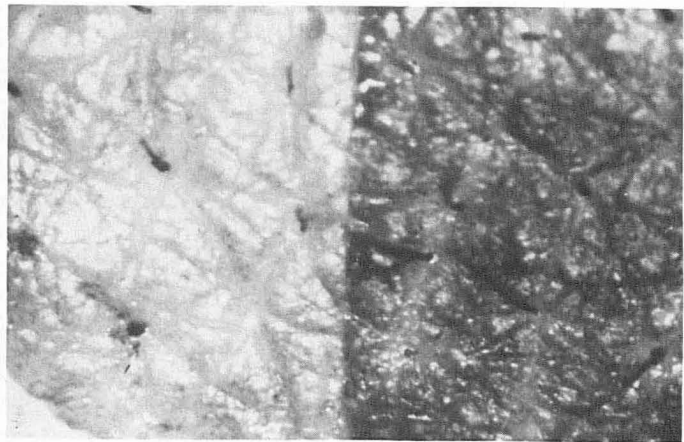


Figure 1. Immediate pigment darkening in vitro. Shave biopsy specimen with intense IPD reaction (right half) after exposure to UVA (40 J/cm²). The left half of the specimen, which was covered during irradiation, shows normal skin color. Epiluminescence photomicrograph.

and the contours of melanocytes and their dendrites were marked. Quantitative analysis of surface and volume densities of melanocytes as well as number and distribution patterns of melanosomes and melanosome complexes was performed by using a computer-assisted image analysis system adapted for this specific purpose.

A detailed description of this procedure will be published separately. In brief, the micrographs were processed digitally by a high resolution TV camera [14] and were then subjected to gray level image segmentation on the monitor of an electronic image analyzer (Quantimet 720, Cambridge Instruments, with DEC PDP-11/34 and VAX-750) [15]. The data of melanosomal surface, circumference, and diameter were then automatically transferred to a computer programmed to analyze melanosomal size distribution and to determine the number of melanosomes per unit volume of keratinocytes and melanocytes [16]. Only fully melanized (stage IV) melanosomes were evaluated. Melanosome complexes were recognized and quantified with the cluster recognition method by means of the partition procedure using the mean spatial distribution of melanosomes [17], and, in addition, with the procedure of local dilatation of single particles after transformation of the picture into a binary-valued image [18].

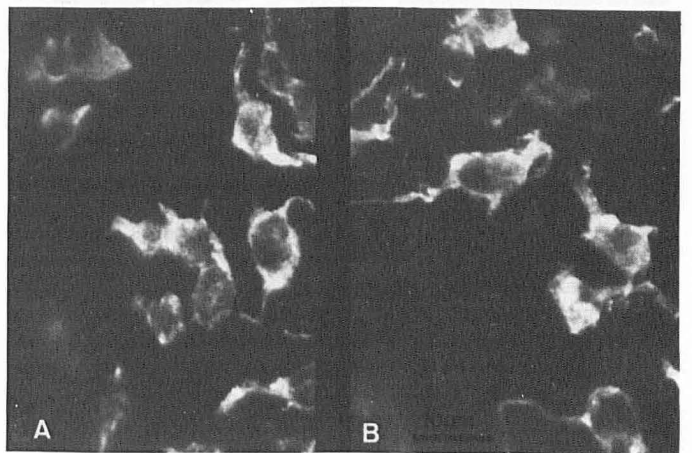


Figure 2. Immunofluorescent staining for the demonstration of vimentin filaments. Epidermal sheet preparation. Melanocytes exhibit a bright fluorescence pattern. No difference between nonirradiated (A) and IPD skin (B) can be observed. × 1000.

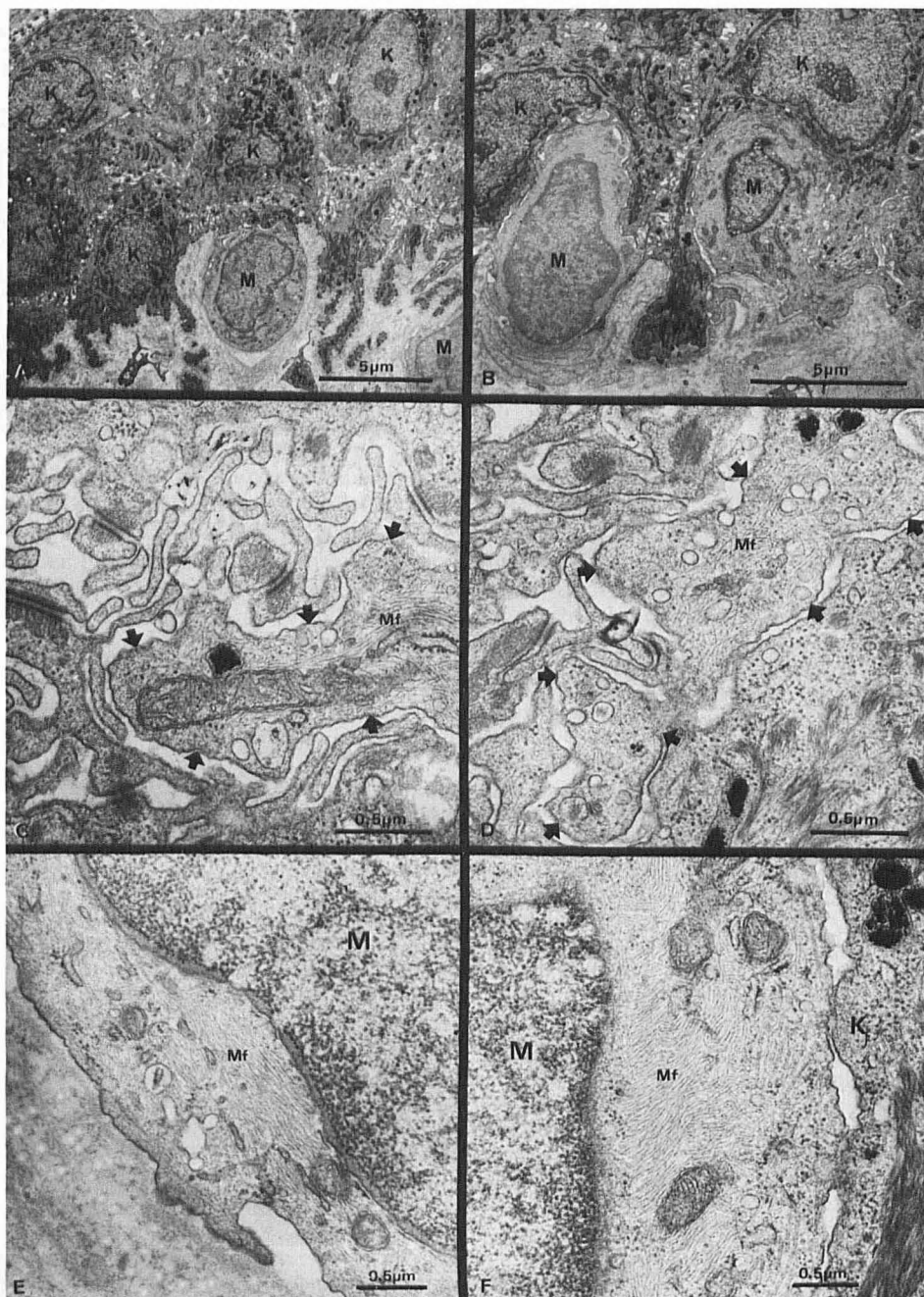


Figure 3. Electron microscopic investigation. Nonirradiated control skin (A,C,E) and adjacent IPD skin (B,D,F) in the same subject. Low magnification (A,B) does not disclose differences with regard to melanosomal distribution in keratinocytes (K) and melanocyte (M) structure. Note marked nuclear capping in the nonexposed specimen (A). Dendrites of melanocytes (arrows) (C,D) surrounded by keratinocytes exhibit an equal distribution pattern of microfilaments (Mf). Also the number of melanosomes within the cytoplasm of the dendrites is similar in both specimens. Microfilament bundles (Mf) show identical distribution and orientation pattern in the perinuclear region of melanocytes (M) of both skin areas (E,F).

RESULTS

In Vitro Studies

Immediate Pigment Darkening Production: Immediate pigment darkening could be elicited in vitro in full-thickness skin (Fig 1) and in epidermal sheet preparations with broad-band UVA radiation. Its production appeared to be independent of the environmental temperature as there was no difference in the degree of coloration whether the medium was kept at 0° or at 32°C. As expected from the in vivo situation, UVB and IR radiation were ineffective with the irradiations used.

Blocking of IPD: Disruption of microfilaments by incubation with cytochalasin B, and of microtubules with colcemid and vincristine did not alter the skin's ability to develop IPD as compared with untreated skin.

Freezing and thawing of the specimens up to 5 times and short-term fixation (30 min) [15] with 5% formalin prior to UVA

exposure also did not inhibit IPD development. However, no IPD could be elicited in specimens fixed for 5 h and 16 h.

Reversibility of IPD: Immediate pigment darkening proved to be reversible after incubation of the specimens for 48 h in tissue culture medium (MEM) in the dark, but only in undamaged skin. Fixation or repeated freezing and thawing inhibited the return to normal of the skin color.

In Vivo Studies

Immunofluorescent Studies: With the antivimentin antibody no morphologic alterations in the vimentin (intermediate) filament system of melanocytes were observed after irradiations of UVA that induced clearly visible IPD (Fig 2A). The nonirradiated control skin exhibited a fluorescence pattern of the cells and their dendrites that was indistinguishable from that of IPD skin (Fig 2B). Changes in structural systems other than the vimentin system cannot be detected with this technique.

Electron Microscopy: Ultrastructural examination did not reveal significant differences between irradiated and nonirradiated skin sites: there was no elongation of melanocytic dendrites, and changes of distribution and/or an increased transfer of melanosomes during the IPD reaction were not observed. Melanosomes or melanosome complexes in keratinocytes in IPD skin were either dispersed throughout the cytoplasm or arranged as a "nuclear cap" and did not significantly differ from controls in their number and distribution pattern (Fig 3A,B). In both nonirradiated skin and IPD skin the melanocytes consistently contained intermediate filament bundles in their dendrites (Fig 3C,D) as well as in the perinuclear regions (Fig 3E,F), and there was no filament aggregation that could have been ascribed to an irradiation effect.

Ultrastructural Morphometry: Morphometric analysis confirmed the results obtained by pure visual assessment. No statistically significant differences with regard to melanosomal size distribution and to the number of melanosomes and complexes per unit volume in keratinocytes and melanocytes were exhibited in IPD and nonirradiated control skin.

DISCUSSION

Previous ultrastructural studies of human skin during the IPD reaction have suggested that IPD represents a dynamic process involving changes in the distribution pattern of melanosomes mediated by a redistribution of the cytoskeletal elements within the melanocyte [6,7]. It has been assumed that both microfilaments and microtubules play an important role in the elongation of melanocyte dendrites and in the transfer of melanosomes into surrounding keratinocytes [6-8]. Thus, the color change during UVA irradiation has been ascribed not only to an immediate oxidation reaction of melanin precursors but also to an active participation of the viable melanocyte.

We became interested in the IPD reaction when we reviewed the early literature in which it was shown that IPD or "Pigmentdunkelung" can be produced in isolated skin specimens taken from corpses by irradiation with a filtered Quartz lamp [1,2], an observation which indicated that IPD may occur independent from the state of viability of the skin.

The experiments performed in the present study were designed to test whether viability is a prerequisite for IPD and whether morphologic changes, if any, can be assessed quantitatively.

In accordance with previous studies [3-5] only UVA irradiation was effective in producing IPD. However, we did not attempt to use UVB irradiations above 100 mJ/cm² since, with large exposures to a broad-band UVB source, the UVA contamination may induce some IPD. Preliminary experiments with high-dose monochromatic radiation at 297 nm (data not shown) failed to reveal any IPD activity.

The observation that IPD occurs at 0°C, after disruption of the filamentous and tubular system by chemical agents, after repeated freezing and thawing, and even after short fixation with 5% formalin indicated that IPD does not depend on the structural or functional integrity of the melanocyte and thus occurs independently of the movement of melanosomes within the cells. Interestingly, the IPD reaction proved to be reversible only under physiologic conditions in undamaged skin. Thus, the reversal of IPD may not be mediated by a simple chemical redox system but requires active involvement of the epidermal cells.

As yet we have no explanation why long-term fixation prevents IPD formation, but one could speculate that prolonged fixation causes major chemical changes in the cells.

After examination of a large number of electron microscopic specimens we found virtually no difference between UVA-irradiated and nonirradiated skin. All morphologic changes and details that had been described by previous authors as representing the structural substrate for IPD formation were present in both groups of specimens. In other words, no IPD-specific changes were encountered. This discrepancy with previous reports could perhaps be explained by the known fact that pure visual assess-

ment of electron micrographs does not allow a reliable quantitative analysis of subtle changes.

Consequently, to avoid our own possible bias we employed morphometric methods that permit a much more objective quantification. Ultrastructural morphometry revealed no changes in the melanocytes or in melanosomal distribution pattern, and this is in keeping with another recent study on quantitative electron microscopy of the IPD reaction [19].

Immediate protection against UV radiation damage would be a quite logical, perhaps atavistic function of IPD, and it is commonly accepted that the amount of melanin in the keratinocytes determines the magnitude of protection. Our observation that IPD does not lead to an increased melanosome transfer into keratinocytes is thus very important in this respect. Willis et al [20], and more recently Black et al [21], could not find a photoprotective effect against UVB erythema by IPD, and preliminary studies from this laboratory indicate that IPD does not protect from UVB-induced DNA lesions that can be detected by measuring unscheduled DNA synthesis.

In summary, IPD is presumably based on a passive, acellular photochemical reaction, perhaps on oxidation and/or polymerization of melanin precursors, or even other constituents of the epidermis. In view of the well-known rapid fading of IPD, true melanogenesis does not seem to be a decisive event. However, the fading, or more precisely, reversibility, of IPD is confined to viable skin and this indicates an active cellular process.

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